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Biochemical analysis of the stress protein response in human oesophageal epithelium

D Hopwood, S Moitra, B Vojtesek, D A Johnston, J F Dillon, T R Hupp

Abstract

Background—The oesophageal epithelium is exposed routinely to noxious agents in the environment, including gastric acid, thermal stress, and chemical toxins. These epithelial cells have presumably evolved effective protective mechanisms to withstand tissue damage and repair injured cells. Heat shock protein or stress protein responses play a central role in protecting distinct cell types from different types of injury.

Aim—To determine (i) whether biochemical analysis of stress protein responses in pinch biopsy specimens from human oesophageal epithelium is feasible; (ii) whether undue stresses are imposed on cells by the act of sample collection, thus precluding analysis of stress responses; and (iii) if amenable to experimentation, the type of heat shock protein (Hsp) response that operates in the human oesophageal epithelium.

Methods—Tissue from the human oesophagus comprised predominantly of squamous epithelium was acquired within two hours of biopsy and subjected to an *in vitro* heat shock. Soluble tissue cell lysates derived from untreated or heat shocked samples were examined using denaturing polyacrylamide gel electrophoresis for changes in: (i) the pattern of general protein synthesis by labelling epithelial cells with ³⁵S-methionine and (ii) the levels of soluble Hsp70 protein and related isoforms using immunochemical protein blots.

Results—A single pinch biopsy specimen is sufficient to extract and analyse specific sets of polypeptides in the oesophageal epithelium. After *ex vivo* heat shock, a classic inhibition of general protein synthesis is observed and correlates with the increased synthesis of two major proteins of molecular weight of 60 and 70 kDa. Notably, cells from unheated controls exhibit a "stressed" biochemical state 22 hours after incubation at 37°C, as shown by inhibition of general protein synthesis and increased synthesis of the 70 kDa protein. These data indicate that only freshly acquired specimens are suitable for studying stress responses *ex vivo*. No evidence was found that the two heat induced polypeptides are previously identified Hsp70 isoforms. In fact, heat shock results in a reduction in the steady state concen-

trations of Hsp70 protein in the oesophageal epithelium.

Conclusion—Systematic and highly controlled studies on protein biochemistry are possible on epithelial biopsy specimens from the human oesophagus. These technical innovations have permitted the discovery of a novel heat shock response operating in the oesophageal epithelium. Notably, two polypeptides were synthesised after heat shock that seem to differ from Hsp70 protein. In addition, the striking reduction in steady state concentrations of Hsp70 protein after heat shock suggests that oesophageal epithelium has evolved an atypical biochemical response to thermal stress.

(Gut 1997; 41: 156-163)

Keywords: oesophagus; heat shock; Hsp70; hyperthermia; stress responses; cancer

The human oesophageal epithelium can be exposed daily to gastric contents, temperature fluctuations, mechanical injury, toxins, and environmental chemicals. Although the effects of most stressful agents on epithelial cell integrity have not been clarified, the damaging effects of refluxed gastric contents are well documented.¹⁻³ Damage induced by reflux of gastric contents seems to be an initiation event in the development of ulceration, Barrett's oesophagus and dysplasia.⁴ Persistent exposure of the oesophagus to a low pH environment in animal models correlates with the conversion of squamous epithelium to columnar epithelium,⁵ but the biochemical mechanisms whereby cell proliferation rates of normal epithelium increase are not known. An important sequence preceding malignancy may be perturbation of the cell cycle regulatory machinery, as one genetic defect frequently observed in Barrett's oesophagus is mutation in the cell cycle checkpoint regulator, p53.⁶ Mutations in the p53 gene often correlate with tetraploidy and increased G2 populations in cells derived from biopsy specimens of Barrett's oesophagus, suggesting that mutation in p53 may have a role in the evolution of tumours in the oesophagus.⁷

Less is known about stresses other than acid reflux, including heat, ethanol, carcinogens, and physical injury, which potentially damage normal oesophageal epithelium. Presumably, oesophageal epithelial cells have evolved effective molecular mechanisms with which to protect and repair cells injured by exposure to environmental damaging agents. For example,

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in animal models, the chemoprotective effects of dietary anticarcinogens in the oesophagus may occur by enhancement of the glutathione S-transferase detoxification system.⁸ Nevertheless, the biochemical response of normal oesophageal epithelium to common types of stress has not been investigated. In contrast, general mechanisms whereby unicellular organisms and tissue culture cells respond to stress have been well studied in the past three decades.

Exposure of proliferating cells to a wide range of damaging agents induces a set of regulatory proteins including kinases/phosphatases, transcription factors, drug metabolising enzymes, cell cycle regulatory proteins, and a highly conserved family of protective or repair polypeptides called heat shock proteins (Hsp). The heat shock proteins are one of the most evolutionarily conserved family of proteins known and include those of molecular weights 25, 40, 60, 70, 90, and 110 kDa.⁹ These polypeptides are involved in regulation of protein conformation and in particular can modulate protein folding, refolding, or unfolding during normal metabolism and following cellular stresses that typically damage proteins.¹⁰ Notably, overexpression of individual members of this stress protein family can confer survival to cells after a normally lethal level of exposure to a toxin or chemical.^{11, 12} It is also important to point out that the heat shock proteins are also induced by agents other than heat, such as ethanol, heavy metals, mutagens, and non-ionising radiation. These data highlight an important role for the heat shock protein family in protection of cells from damage or repair of injured cells and their potential therapeutic role.¹³

The biochemical mechanisms whereby cells respond to heat shock or environmental stress in general are beginning to be unravelled. In most cells studied to date, two specific events can be detected following heat shock. One is the inhibition of general protein synthesis and the second is the specific synthesis of a major heat shock protein, usually Hsp70.¹⁰ In theory, as the human oesophageal epithelium is exposed to damaging agents, these cells provide a good model system for studying: (1) fundamental signal transduction events regulating stress protein responses; (2) the types of adaptive responses which protect cells from injury following exposure to chemicals or thermal stress; and (3) biochemical mechanisms contributing to metaplasia and cancer. However, it is not certain that biochemical studies are possible on small biopsy samples nor is it certain that biopsied tissue remains unstressed *ex vivo*. Cellular injury *ex vivo* would complicate analysis of normal stress responses and preclude the use of human oesophageal epithelium as a model system. We describe a system which defines the basic stress protein responses in human oesophageal epithelium, with particular reference to whether *ex vivo* analysis of stress protein responses are actually possible using pinch biopsy specimens comprised primarily of squamous epithelial cells, and if so, whether: (i) general protein synthesis is af-

fects by heat shock; (ii) induction of the classic heat shock protein Hsp70 occurs; and (iii) the types of proteins synthesised after heat shock are typical of previously studied cell types.

Methods

PATIENTS

Oesophageal biopsy specimens from endoscopically and histologically normal tissue were obtained from the middle third of the oesophagus with informed consent from patients attending an endoscopy clinic for upper gastrointestinal diseases. Human oesophageal epithelium derived from biopsy specimens has been previously characterised *in vitro*.¹⁴ The tissue is composed primarily of squamous epithelial cells (95%) and other cell types, mostly mesenchymal, but also Langerhans cells, T lymphocytes, and small round cells.¹⁵ Morphological integrity is maintained *in vitro* up to 24 hours after harvesting into organ culture media and endocytosis of fluorescent microspheres remains intact for up to six hours *in vitro*.¹⁶ We analysed samples from 27 patients (13 men and 14 women; age range 26–59 years). The study was approved by the Tayside Medical Ethical Committee.

HEAT TREATMENT OF SAMPLES

Biopsy specimens were collected in HAMS-F10 media (Gibco-BRL, Paisley, UK) prechilled to 0°C. Within two hours of biopsy, individual samples were transferred to a small plastic polyethylene dish containing 200 µl HAMS-F10 media preincubated in a 37°C incubator with 5% CO₂ and then immediately incubated in 200 µl HAMS-F10 media for 20 minutes at either 37, 45, or 55°C. Then, samples were incubated in 200 µl HAMS-F10 media at 37°C for various times and were either immediately frozen in liquid nitrogen or pulse labelled with radiolabelled methionine as described later. Some biopsy samples were transferred straight from the biopsy forceps into liquid nitrogen.

CHEMICAL TREATMENT OF SAMPLES

Biopsy specimens were prepared as described for heat treatment, but were exposed to acidic media (pH 3.65), ethanol (4% w/v), or sodium arsenite (500 µM).

LYSIS OF BIOPSY SAMPLES

Lysis buffer (containing 1% NP-40, 25 mM HEPES (pH 7.6), 5 mM DTT, 0.4 M KCl, 5 mM EDTA, 2 µg/ml Perfabloc, 20 µg/ml leupeptin, 1 µg/ml aprotinin, 2 µg/ml pepstatin, 10 µg/ml trypsin inhibitor, and 1 mM benzamide) was added to frozen samples, which were lysed by homogenisation using a hand-held pestle. Following incubations at 0°C for 20 minutes, the suspension was centrifuged at 14 000 rpm (0°C) for 10 minutes in a microcentrifuge. The soluble supernatant was recovered and protein concentration was determined by Bradford's method.¹⁷

RADIOLABELLING OF PROTEIN IN BIOPSY SAMPLES

After biopsied samples were prepared and incubated as described above, the cells were

labelled by replacing HAMS-F10 media with labelling media (methionine+cysteine-free DMEM (media from ICN), containing 10% dialysed fetal calf serum, 2 mM glutamine, and 100 μ C/ml 35 S-methionine) and incubating for an additional 45 minutes at 37°C. Samples were frozen in liquid nitrogen and stored at -70°C prior to lysis. It was important to label the cells in vitro with radioactive methionine for very short time periods (less than 60 minutes), as prolonged incubations using radioactive amino acids have been recently shown to induce a radiation damage response pathway.¹⁸

DENATURING GEL ELECTROPHORESIS AND FLUOROGRAPHY

Indicated amounts of epithelial lysates were electrophoresed on SDS-denaturing polyacrylamide gels (10% or 12% polyacrylamide). Steady state concentrations of protein were analysed by staining gels with Coomassie blue. Protein synthesis was analysed in radiolabelled cells by impregnating the polyacrylamide gels with Amplify (Amersham) after electrophoresis and exposing the dried gel to x ray film for seven to 21 days at -70°C.

IMMUNOCHEMICAL METHODS

To detect Hsp70 protein and related isoforms using western blotting techniques, 10 μ g total cell lysate was applied to a polyacrylamide gel, bound protein was transferred to nitrocellulose (Amersham), and immunoreactive protein was detected using a monoclonal antibody to Hsp70 protein (SPA-710, Stratagene) or using a novel monoclonal antibody obtained by standard fusion from mice immunised with Hsp70 protein. This new monoclonal antibody (MB-H1) cross-reacts with Hsc70 and related isoforms (see fig 6) and can be obtained from Biovation (Aberdeen, Scotland).

Results

RAPID PERTURBATION IN THE STEADY STATE CONCENTRATIONS OF A MAJOR POLYPEPTIDE IN HUMAN OESOPHAGEAL EPITHELIUM EX VIVO

As stress protein responses can be activated by distinct types of environmental perturbations, it was first important to define whether ex vivo organ culture would impose an undue stress on the epithelial cells, thus precluding biochemical analysis of the heat shock response. Although no morphological changes were observed after organ culture ex vivo, this does not reflect whether molecular stress response pathways are activated. The assay used to analyse biochemical integrity relied on examination of the pattern of steady state protein concentrations by visualisation of polypeptides derived from lysates of a single pinch biopsy specimen (quickly frozen or collected into media) using denaturing polyacrylamide gel electrophoresis. It is of interest to note that approximately 100 μ g soluble protein can be extracted from a single pinch biopsy sample (data not shown). This provides material for at least 10 biochemical experiments and establishes the feasibility of studying enzymatic processes using human oesophageal epithelium.

Biopsied material derived from normal tissue and quickly frozen in liquid nitrogen exhibits the steady state pattern of protein concentrations shown in fig 1 (lane 1). Collection of the tissue onto ice for subsequent study ex vivo results in a prominent reduction in concentrations of one major polypeptide, called Protein a (molecular weight of 70 kDa, indicated by arrow). In addition, incubation of the tissue samples for 20 minutes at temperatures from 37 to 55°C in a media bath also results in a more pronounced reduction in the concentrations of Protein a. These results indicate that, although an (at present) undefined stress is imposed upon this tissue ex vivo, dramatic changes in protein biosynthesis are not evident. With these variables defined we examined the effects of in vitro heat shock on the stress protein response in oesophageal epithelium.

TWO PREDOMINANT POLYPEPTIDES ARE SYNTHESISED AFTER EX VIVO HEAT SHOCK IN THE OESOPHAGEAL EPITHELIUM

The following approach involved examination of changes in the rates of protein synthesis without or after a 20 minute heat shock in order to determine the type of heat shock response operating in the oesophageal epithelium. Oesophageal biopsy samples were exposed to temperatures of 37, 45, or 55°C for 20 minutes, followed by a recovery phase at 37°C for four hours. At this time point, the pattern of protein synthesis was analysed by pulse labelling the tissue with 35 S-methionine for 45 minutes and processing soluble protein lysates using denaturing polyacrylamide gel electrophoresis and fluorography.

In the absence of heat shock, a reproducible pattern of protein synthesis is observed amongst three representative patients (fig 2; lanes 1, 4, and 7). Following exposure of the

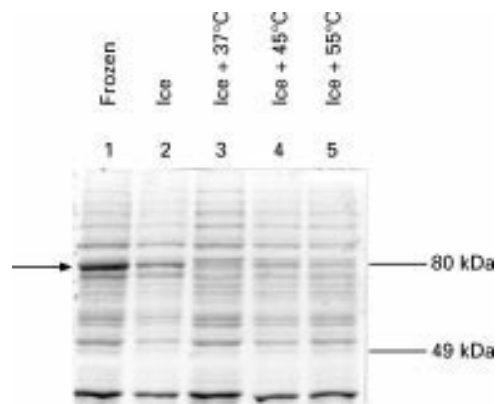


Figure 1: Ex vivo steady state protein concentrations derived from tissue biopsy samples. Soluble protein (10 μ g) derived from tissue lysates was applied to a 10% SDS-polyacrylamide gel and total protein was detected by staining with Coomassie blue. Before lysis, the tissue samples were treated as follows: lane 1, immediately frozen; lane 2, incubated in media at 0°C before freezing; lanes 3-5, transferred from 0°C to media at 37°C and then immediately incubated for 20 minutes at either 37°C (lane 3), 45°C (lane 4), or 55°C (lane 5). Then, samples were incubated at 37°C for four hours and were immediately frozen in liquid nitrogen. The arrow shows the migration of a predominant polypeptide whose concentrations were dramatically reduced ex vivo.

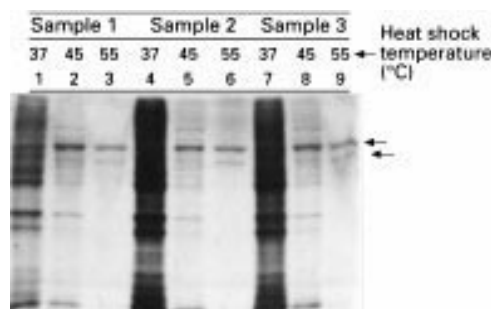


Figure 2: Protein synthesis in tissue biopsy samples after an *in vitro* heat shock at different temperatures. Samples from three representative patients (samples 1–3) were incubated for 20 minutes at 37°C (lanes 1, 4, and 7), 45°C (lanes 2, 5, and 8), or 55°C (lanes 3, 6, and 9), and then after four hours of equilibration at 37°C, the samples were incubated in fresh media containing ^{35}S -methionine for an additional 45 minutes at 37°C. Radiolabelled protein derived from cell lysates was visualised using SDS-polyacrylamide gel electrophoresis and fluorography. The arrows show the migration of the two major polypeptides synthesised after heat shock.

epithelium to elevated temperatures, there is a progressive inhibition of the “normal” pattern of protein biosynthesis (lanes 2, 5, and 8) with a concomitant increase in the relative synthesis of two major polypeptides after exposure to the most severe heat shock (lanes 3, 6, and 9; see arrows). The inhibition of general protein biosynthesis with the corresponding increase in synthesis of specific polypeptides is characteristic of a cellular response to stressful levels of heat.

LIMITATIONS ON THE USE OF EPITHELIAL CELLS FOR STRESS RESPONSE STUDIES EX VIVO

The experiments above examined stress protein responses within four hours after culturing the epithelium. The pattern of protein synthesis in unstressed cells was next examined after prolonged incubation in organ culture media to determine: (i) whether cells eventually induce a stress protein response *ex vivo* in the absence of overt injury, and (ii) if so, whether the proteins induced are similar to or different from the polypeptides synthesised after heat shock.

A time course of inhibition of general protein biosynthesis and simultaneous increase in synthesis of heat shock proteins is shown in fig 3. Ranging from 30 minutes (data not shown) to 22 hours after a 20 minute heat shock at 55°C (fig 3; lane 5 *v* lane 6), the two major proteins being synthesised are those of molecular weight of 60 and 70 kDa, indicating that the epithelial cells respond very rapidly and for prolonged periods of time to a relatively short heat shock.

Although the 70 kDa protein is absent in non-heated samples four hours after sample processing (fig 3; lanes 1 and 3), this protein is synthesised in unheated samples 22 hours after processing *ex vivo* (fig 3, lane 5). In addition, these cells are beginning to shut down general protein synthesis to a large extent. These results indicate that although heat stress is required to induce the synthesis of the 70 kDa protein rapidly, an undefined biochemical stress operates on the unheated epithelial cells many hours after sample collection. These latter results highlight the fact that the epithelial

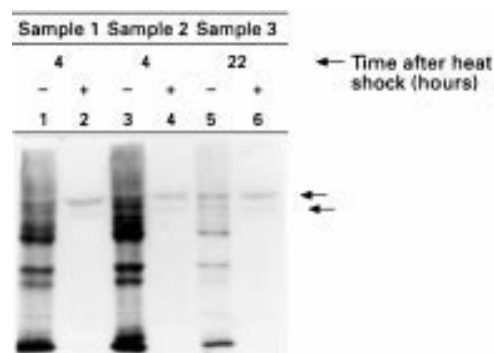


Figure 3: Prolonged incubation of tissue biopsy specimens induces a stress protein response in the absence of heat shock. Samples from three representative patients (samples 1–3) were incubated for 20 minutes at 37°C (lanes 1, 3, and 5) or 55°C (lanes 2, 4, and 6), and then after four hours (lanes 1–4) or 22 hours (lanes 5 and 6) of equilibration at 37°C, the samples were incubated in fresh media containing ^{35}S -methionine for an additional 45 minutes at 37°C. Radiolabelled protein derived from cell lysates was visualised using SDS-polyacrylamide gel electrophoresis and fluorography. The arrows show the migration of the two major polypeptides synthesised after heat shock.

cells eventually induce stress protein responses *ex vivo* and that fairly rapid analysis is required following tissue biopsy to maintain cells in a relatively unperturbed state. It is also interesting to note that the stress proteins being synthesised after 22 hours in unheated samples seem to be similar to those induced after heat shock, suggesting that distinct signalling pathways can induce similar stress proteins.

HSP70 PROTEIN CONCENTRATIONS DECREASE AFTER HEAT SHOCK IN HUMAN OESOPHAGEAL EPITHELIUM

Studies were subsequently initiated in order to acquire information as to the nature of the two heat induced polypeptides in human oesophageal epithelium. Given that one of the major heat induced proteins in many cell types is the heat inducible form of the stress 70 family of proteins (Hsp70), changes in the concentrations of Hsp70 protein were examined in soluble lysates derived from human oesophageal epithelium using a monoclonal antibody which is specific for this heat inducible Hsp70 isoform (fig 4).

Similar basal concentrations of Hsp70 protein can be detected in three different samples four hours or 22 hours after sample collection (fig 4, lanes 4, 6, and 8, respectively). Interestingly, four or 22 hours after heat shock at 55°C, during which oesophageal epithelial cells exhibit the synthesis of two major proteins depicted in fig 3, there is a substantial decrease in the concentrations of soluble Hsp70 protein (fig 4, lanes 3, 5, and 7). This atypical response to thermal stress suggests that either (i) Hsp70 protein is not being synthesised after heat shock; or (ii) Hsp70 protein is being synthesised but is preferentially degraded after heat shock; or (iii) Hsp70 protein is being synthesised after heat shock but enters into an insoluble pool. Future work will tackle the mechanism underlying this atypical phenomenon.

As a control establishing the integrity of our reagents and to show a typical biochemical response to heat shock in mammals, an

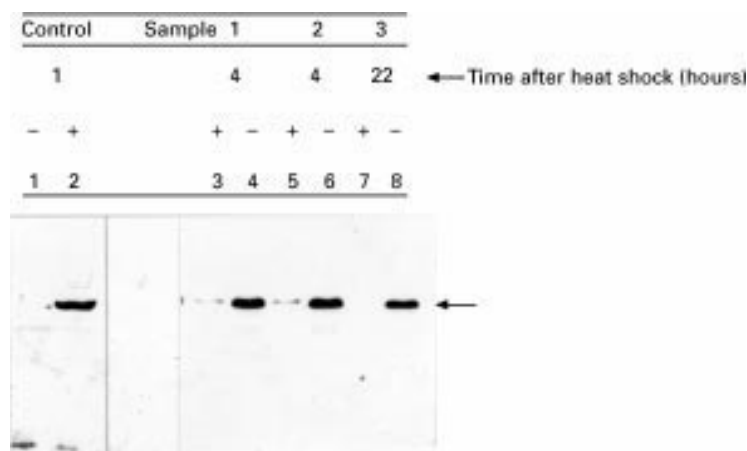


Figure 4: Hsp70 protein concentrations decline after heat shock. Biopsy samples from three representative patients (samples 1–3) were incubated for 20 minutes at 37°C (lanes 4, 6, and 8) or 55°C (lanes 3, 5, and 7), and then after four hours (lanes 3–6) or 22 hours (lanes 7 and 8) of equilibration at 37°C, the samples were incubated in fresh media containing 35 S-methionine for an additional 45 minutes at 37°C. Hsp70 protein derived from soluble cell lysates (10 μ g) was visualised using SDS-polyacrylamide gel electrophoresis and immunoblotting with a monoclonal antibody specific for Hsp70 protein. As a control, the concentrations of Hsp70 protein were measured in soluble lysates from normal (10 μ g; lane 1) or heat shocked lung (lane 2; one hour after a mild hyperthermic induction in male Wistar rats). The arrows show the migration of Hsp70 protein.

increase in concentrations of Hsp70 protein can be observed in rat lung following whole body hyperthermia (fig 4; lane 1 *v* lane 2 (and many other tissues, Vojtesek and Hupp, manuscript in preparation)), indicating that our reagents can be used to detect Hsp70 protein accumulation after heat shock in another mammalian system.

We finally investigated the concentrations of Hsp70 protein in samples which had been subjected to progressively elevated levels of heat shock (fig 5). With increasing temperature from 37 (lane 3) to 45 (lane 2), to 55°C (lane 1), there is a dose dependent decrease in the concentrations of Hsp70 protein, indicating that the decrease in Hsp70 protein concentrations correlates with the degree of thermal stress.

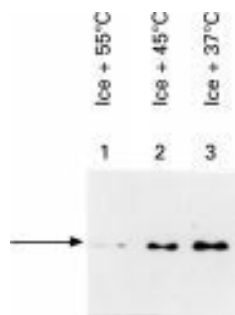


Figure 5: Progressive decrease in Hsp70 protein concentrations with increasing thermal stress. Tissue biopsy samples were treated as follows: the cells were transferred from 0°C to media at 37°C and then incubated for 20 minutes at either 37°C (lane 3), 45°C (lane 2), or 55°C (lane 1). Then, samples were incubated at 37°C for four hours and were frozen in liquid nitrogen. Soluble protein (10 μ g) derived from tissue lysates was applied to a 10% SDS-polyacrylamide gel and Hsp70 protein concentrations were quantified using SDS-polyacrylamide gel electrophoresis and immunoblotting with a monoclonal antibody specific for Hsp70 protein. The arrow shows the migration of Hsp70 protein.

HSC70 PROTEIN DOES NOT ACCUMULATE AFTER HEAT SHOCK IN HUMAN OESOPHAGEAL EPITHELIUM

Although soluble Hsp70 protein concentrations do not increase following heat shock, there are at least six putative isoforms of the stress 70 family of proteins (including the constitutively induced family member, Hsc70⁹) that may be regulated in a cell type specific manner. As such, we generated a monoclonal antibody to human Hsp70 protein using standard methods¹⁹ that could cross-react with a broad range of stress 70 family members from different cell types in order to examine oesophageal epithelium for alterations in general stress 70 protein concentrations before and after heat shock.

One of our new monoclonal antibodies (MB-H1) could detect purified human Hsp70 protein (fig 6, lane 5), purified human Hsc70 (fig 6, lane 6), Hsc70, and Hsp70 protein derived from four different human tumour cell lines (fig 6, lanes 1–4), and two Hsp70 isoforms derived from rat testes²⁰ (data not shown). Thus, this antibody seems to be a good reagent

with which to examine concentrations of general stress 70 family members.

Using the same sample material as in fig 4, there are no pronounced increases in the concentrations of Hsc70 isoforms using monoclonal antibody MB-H1 (fig 7). These results indicate that, although two major proteins in the molecular weight range of 60 and 70 kDa are preferentially synthesised after heat shock, they do not correlate with increases in steady state concentrations of Hsp70 or stress 70 protein isoforms.

EFFECTS OF ACID, ETHANOL, AND ARSENITE STRESS ON THE HEAT SHOCK PROTEIN RESPONSE

Having designed two distinct assays for examining the heat shock protein response in oesophageal epithelium, we examined the effects of perturbations other than heat shock on protein synthesis and Hsp70 protein concentrations. A relatively short exposure of epithelial cells to ethanol (4%) or low pH (pH 3.65) conditions did not alter general protein synthesis (fig 8, left panel, lanes 1–3) or Hsp70 protein concentrations (fig 8, right panel, lanes 1–3).

As arsenite has also been reported to affect cellular metabolism in a manner similar to hyperthermia, the effects of arsenite incubation on stress protein synthesis were also examined. As observed with heat shock, arsenite (500 μ M) inhibited general protein synthesis with the predominant synthesis of the 68 kDa polypeptide (data not shown). In summary, two stresses can mimic thermal stress; (1) arsenite, and (2) prolonged organ culture (fig 3, lane 5). These results suggest that the novel heat shock proteins may be controlled by different signalling pathways that recruit the same effector polypeptides.

Discussion

THE HEAT SHOCK RESPONSE IN HUMAN OESOPHAGEAL EPITHELIUM

One of two biochemical events that typically follow exposure of cells to severe forms of heat shock is the inhibition of general protein synthesis.⁹ As observed in many other cell types, exposure of oesophageal epithelium to increasing levels of heat stress gives rise to increasing inhibition of general protein synthesis (fig 2). Such studies are possible only due to the fact that biopsied oesophageal epithelium is not unduly stressed *ex vivo* early after sample collection. However, prolonged studies are not feasible *ex vivo* as 22 hours after sample collection, unheated oesophageal epithelium induces the synthesis of the two new heat shock proteins with concomitant inhibition of general protein synthesis (fig 3). These data also show that non-heat shock signalling pathways can induce these two novel heat shock proteins, suggesting that oesophageal epithelial cells may harbour the machinery to respond to diverse stimuli by inducing the same sets of stress proteins.

The biochemical mechanisms whereby heat injury to a cell modulates the activity of the translational machinery are not yet clear. One likely mediator, however, is the ribosome

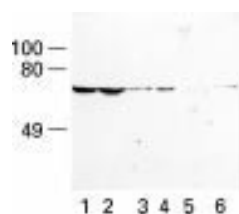


Figure 6: Characterisation of a novel monoclonal antibody to Hsp70 protein isoforms. Protein immunoblots using a novel monoclonal antibody generated to Hsp70 protein (MB-H1) were performed with purified Hsc70 (lane 5), purified Hsp70 (lane 6) and the Hsp70/Hsc70 isoforms in four distinct tumour cell lines (lanes 1–4; BT549 (breast cancer); HS913T (fibrosarcoma); SK-UT-1 (leiomyosarcoma); and MCF7 (breast cancer), respectively).

translation factor, eIF-2. Signal transduction mechanisms also seem to play a part in regulating the activity of eIF-2 as inhibition of protein synthesis upon exposure of cells to heat shock correlates with the phosphorylation and inactivation of eIF-2 alpha function,²¹ indicating the importance of phosphorylation in the control and regulation of protein synthesis in response to heat shock. However, mutation of the major eIF-2 phosphorylation site to alanine results in only a partial alleviation of heat induced inhibition of protein synthesis, suggesting that another factor(s) is involved in modulating inhibition of protein synthesis.²²

Inhibition of protein synthesis following heat stress in the oesophageal epithelium may not be surprising per se. However, given that prior exposure to thermal stress can protect many cell types from subsequently damaging levels of heat, and that diets of many patients involve the routine intake of hot drink and food, it was thought possible that heat shock in vitro might not effect the metabolism of oesophageal epithelium. For example, in a laboratory trial, the average temperature of ingested liquids is from 55 to 65°C.²³ In our studies, a heat stress from 45 to 55°C does lead to inhibition of protein synthesis in the normal epithelium of every patient examined, suggesting that oesophageal epithelium does not exhibit an unusual resistance to heat shock in vitro. Nevertheless, we are also attempting to identify the major polypeptide, Protein a, whose concentrations decrease dramatically ex vivo after biopsy (fig 1). Preliminary evidence indicates that changes in steady state concentrations of this polypeptide may vary among patients, suggesting that it may be a reliable biochemical marker of stress.

The second of two molecular responses of cells after exposure to severe heat shock is the increased synthesis of a set of proteins, termed stress or heat shock proteins. The major polypeptide usually synthesised corresponds to Hsp70 protein. Although most studies have been done using unicellular organisms, insects,

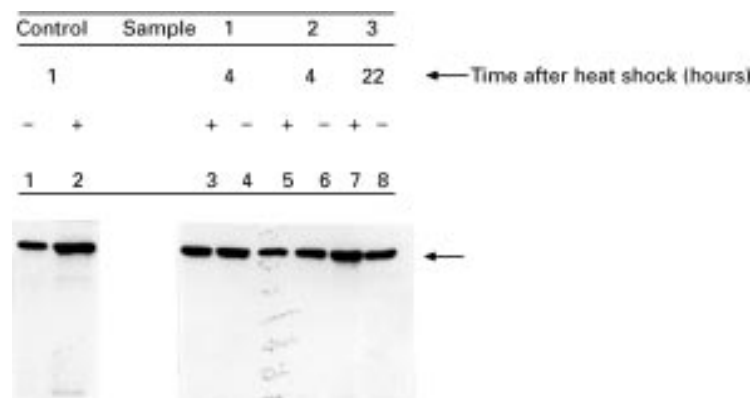


Figure 7: Hsc70 protein concentrations remain constant after heat shock. Biopsy samples from three representative patients (samples 1–3) were incubated for 20 minutes at 37°C (lanes 4, 6, and 8) or 55°C (lanes 3, 5, and 7), then after four hours (lanes 3–6) or 22 hours (lanes 7 and 8) of equilibration at 37°C, the samples were incubated in fresh media containing ³⁵S-methionine for an additional 45 minutes at 37°C. Hsc70 protein and related isoforms derived from soluble cell lysates were visualised using SDS-polyacrylamide gel electrophoresis and immunoblotting with monoclonal antibody MB-H1. As a control, the concentrations of Hsp70 protein were determined in soluble lysates from normal (10 µg; lane 1) or heat shocked lung (lane 2; one hour after a mild hyperthermic induction in male Wistar rats). The arrows show the migration of Hsc70 protein and isoforms.

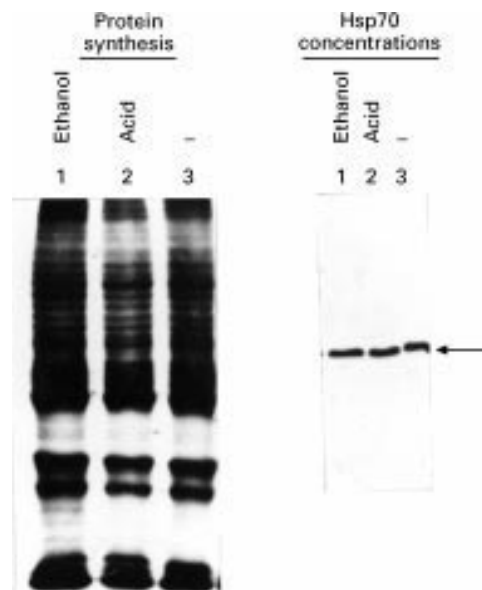


Figure 8: The effects of ethanol and acid exposure on the heat shock protein response. Biopsy samples were incubated for 20 minutes at 37°C in normal media (lane 3), low pH media (lane 2), or 4% ethanol (lane 1). After four hours of equilibration at 37°C in fresh media, the samples were incubated in media containing ³⁵S-methionine for an additional 45 minutes at 37°C. Left panel: radiolabelled protein derived from cell lysates was visualised using SDS-polyacrylamide gel electrophoresis and fluorography. Right panel: Hsp70 protein derived from cell lysates was visualised using SDS-polyacrylamide gel electrophoresis and immunoblotting with a monoclonal antibody specific for Hsp70. The arrow shows the migration of Hsp70 protein.

or mammalian tissue culture systems, there have also been a few key studies performed on the effects of heat shock on Hsp70 protein biosynthesis in some tissues. Analysis of the heat shock response in rat cardiac muscle has clearly shown that the inhibition of general protein synthesis occurs with selective increases in the rates of synthesis of one major protein of molecular weight 70 kDa.²⁴ Exercise can also lead to induction of stress proteins in vivo.²⁵ In addition, in vitro heat shock of tissue biopsy specimens derived from human tumours leads to the increased synthesis of Hsp70 protein.²⁶ In contrast to these studies, we have shown that Hsp70 protein concentrations decrease in normal oesophageal epithelium after heat shock, suggesting that this cell type may have an unusual heat shock response. A similar decrease in concentrations of Hsp70 protein following in vitro heat shock of a prolactin dependent pre-T cell line²⁷ provides one of the few other examples in which Hsp70 protein concentrations actually decrease after thermal stress.

The mechanisms whereby Hsp70 protein concentrations decrease after heat shock are not clear at present. It is interesting to note that concentrations of Hsp70 protein decrease without increases in Hsp70 protein synthesis (fig 4). This is specific for Hsp70 protein, as Hsc70 protein concentrations do not change after heat shock. Thus, one mechanism accounting for decreases in Hsp70 protein concentrations seems to be through selective degradation of Hsp70 protein after heat shock in this cell type.

Other mechanisms may account for the inability to induce Hsp70 protein biosynthesis after stress. Normally after a cellular heat shock, activation of the heat shock transcription factor (HSF) leads to increases in expression of the hsp70 gene, which is coupled to increases in rates of translation of the hsp70 messenger RNA, and leads to an increase in soluble forms of Hsp70 protein. Cellular systems do exist in which Hsp70 protein synthesis is uncoupled from HSF function. For example, Hsp70 protein induction during aging in flight muscle and leg muscle is not dependent upon gene transcription, but on post-transcriptional mechanisms.²⁸ In addition, heat shock of Y79 retinoblastoma cells does not lead to increases in hsp70 gene expression, despite activation of HSF and hsp90 gene transcription.²⁹ These results indicate uncoupling of stress induced activation of hsp70 and hsp90 genes, which are typically coordinately regulated in response to stress. Similarly, ERK1 kinase activation in NIH3T3 cells can result in delayed HSF activation and decreased Hsp70 protein synthesis,³⁰ suggesting that kinases can act as negative regulators of the heat shock response. Based on these latter published precedents and our data, decreases in concentrations of Hsp70 protein in the oesophageal epithelium could relate directly to Hsp70 protein degradation, increased entry into insoluble pools, decreases in activation of HSF, decreases in hsp70 gene expression, decreases in translation, or increases in kinase signalling pathway activation. Clearly, the stress responses are proving to be cell type dependent,³¹ underscoring the necessity of using organ culture to aid in the understanding of physiological responses to damage.

CLINICAL RELEVANCE OF THE HEAT SHOCK RESPONSE IN HUMANS

Although heat shock proteins have been classically discovered as being induced by a thermal stress, they can be induced by a wide range of damaging agents other than heat. In addition, their abnormally elevated levels in humans are thought to reflect tissue injury, damage, or a diseased state. The therapeutic effects of heat shock on organ function have been reported in mammals. Heat shock protein induction has been reported to improve myocardial recovery after ischaemic injury.³² Similar regulatory responses operate in kidney, where heat shock by perfusion can protect the organ against warm ischaemic injury.^{33, 34}

There have been a few reports of thermal stress³⁵ and heat shock protein or stress protein expression in the gastrointestinal tract. Hsp70 protein expression has been recorded in ischaemic bowel in relation to regenerative epithelial and smooth muscle cells.³⁶ Hsp60 protein induction has been demonstrated in a number of liver diseases, such as primary biliary cirrhosis and primary sclerosing cholangitis, whereas others are found in secondary, biliary obstruction and alcohol induced injury.³⁷ Whether the expression of these heat shock proteins is a response to or a component of the disease is not clear at present, and their

role in a number of diseases has been discussed including ischaemia, autoimmune diseases, and malignancy.⁹ Gastric mucosal cells in culture are protected against damage by ethanol if they are given a prior heat shock³⁸ or treatment with Geranylgeranylacetone.³⁹ This transference of protection is similar to that in skin, where heat shock protects against ultraviolet B injury.¹¹ Currently, we are also trying to identify the combined and individual effects of bile or acid on stress protein responses and the protective effects of these stress proteins on protection from bile or acid induced cellular injury.

In conclusion, an atypical HSP response is induced in the human oesophageal epithelium after thermal stress. Although Hsp70 protein or related isoform concentrations do not increase in soluble tissue lysates after heat shock, oesophageal epithelial cells do exhibit the synthesis of two predominant polypeptides that are at present unidentified. Using antibodies to Hsp25, Hsp60, and Hsp90, we have found no evidence that the 60 and 70 kDa proteins comprise these latter family members (data not shown). Future biochemical studies will be required to purify the heat shock proteins synthesised in oesophageal epithelium after heat shock and generate reagents to study their expression in distinct cell types to determine whether their expression is unique or common to general stress responses. For example, we already know (fig 3, lane 5) that prolonged culture is sufficient to induce the 70 kDa protein, suggesting that these cell types have evolved the ability to recruit these stress proteins in response to distinct types of damage. In addition, it remains to be seen whether induced heat shock proteins in oesophageal mucosa will protect against other mechanisms of damage, such as acid and carcinogens, or whether other forms of stress give protection against heat damage. Heat shock proteins not only protect normal cells from injury, but they can block the effects of anticancer drugs or the induction of apoptosis in tumour cells,¹² while they may also regulate apoptosis during normal cell physiology in a tissue specific manner.²⁰ As such, understanding the biochemical basis for the multistep nature of inflammation and cancer development in the human oesophagus will involve studying the expression of stress proteins and the protection they provide normal and diseased cells from injury.

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